On the missing links between the epidemiology and pathophysiology of Staphylococcus aureus

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Chapter 1

General Introduction and Scope

Staphylococcus aureus - discovery and naming

Research on *Staphylococcus aureus*, the Gram-positive bacterium investigated in the present PhD thesis research, started with its isolation from the surgical wound of a patient by the Scottish Surgeon, Sir Alexander Ogston, 138 years ago. He reported *S. aureus* as the etiological agent of suppurative abscesses during the 9th Surgical Congress in Berlin (1880)[1]. The bacterium's genus name, *Staphylococcus,* is derived from the Greek words 'staphyle' ('a bunch of grapes') and 'coccos' ('berry'), describing the microscopic features of organisms belonging to this genus. Its species name '*aureus*' is based on the Latin word 'aurum', which refers to the 'golden' pigmentation first described by Anton J. Rosenbach[2]. Importantly, *S. aureus* is one of the World Health Organization's 2017 priority bacteria that have become a worldwide threat for human health due to their high resistance to the currently available antibiotics[3].

Staphylococcus aureus - an unreliable companion

About a third of the world population carries *S. aureus*, the mucosa and skin being its preferred niches. As such, this Gram-positive bacterium is often regarded as a human commensal. Direct skin-to-skin contact and contact with contaminated objects or surfaces are its main routes of transmission between individuals. Importantly, the ability of *S. aureus* to adjust its physiology to changing conditions, especially to insults by the human innate and adaptive immune defenses determine the outcome of host colonization[4]. Once *S. aureus* has breached barriers and at least partially escaped the host's immune defenses, it is capable of causing a wide array of diseases ranging from simple skin and soft tissue infections to life-threatening diseases, such as severe pneumonia, sepsis, osteomyelitis, toxic shock syndrome, and endocarditis[4,5]. Notably, *S. aureus* is not only a major health problem in humans but also in livestock where it is, for example, a causative agent of mastitis in cows[6–8]. In particular, its high genomic plasticity, driven by mutations or rearrangements of its genome as well as horizontal gene transfer (HGT), enables *S. aureus* to adapt to different host niches. Thus, *S. aureus* is an opportunistic pathogen rather than a commensal and, in case of asymptomatic carriage, it should at least be regarded as an 'unreliable companion'.

Staphylococcal genome expansion - gain is better than loss

In general, HGT can occur through transduction, transformation, or conjugation. Transduction is a key mechanism of HGT by which DNA is transferred from one cell to another through bacteriophages (phages). This process involves integration of the phage-transmitted foreign DNA into the chromosome of a recipient cell that subsequently passes it on to its offspring. DNA molecules with sizes of up to 45 kilo

base pairs (kb) can efficiently be transferred *via* phage-mediated transduction[9]. In addition to phages, *S. aureus* pathogenicity islands (SaPIs) help in the transduction of mobile genetic element (MGEs). SaPIs are in fact MGEs themselves. They are approximately 14-17 kb in size. SaPIs encode an integrase required for phage-induced excision of the SaPI DNA, a Rep protein for replication, and a terminase needed for packing the SaPI DNA into phages[10].

It has recently been proposed that *S. aureus* can also undergo natural transformation. Morikawa and colleagues revealed that the natural competence of *S. aureus* to bind and internalize extracellular DNA, a pre-requisite for transformation, occurs through the activation of SigH, an alternative sigma factor[11]. The authors invoked two distinct mechanisms in the activation of SigH, namely chromosomal gene duplication rearrangements, and post-transcriptional regulation. The former is a rare gene duplication event that generates a new chimeric *sigH* gene leading to stochastic production of the SigH protein. In the case of post-transcriptional regulation, inverted repeat sequences upstream of the translation initiation site prevent *sigH* expression, most probably through the formation of secondary structure that occludes the ribosome-binding site. Yet, this does not happen in all cells of a population, which allows the activation of SigH in a subpopulation. As a result, natural transformation of chromosomal or plasmid DNA will occur[11].

Next to transduction and transformation, a third means of HGT is conjugation, a mechanism that requires direct contact between the donor and recipient cells, and subsequent DNA passage through a pore. To achieve conjugative DNA transfer, *S. aureus* requires *tra* genes encoding proteins necessary for conjugation. Such genes are not present in all strains, as they are mostly located on so-called conjugative plasmids. Unlike transduction, conjugation is efficient in transferring DNA molecules with sizes of more than 45 kb. However, it is believed that conjugation is a less frequent means of HGT in *S. aureus* than transduction[12].

A clear advantage of the acquisition of foreign DNA *via* HGT is the ability for microorganisms, such as *S. aureus*, to acquire genes that allow them to withstand host immune responses and the detrimental effects of toxic compounds, such as antibiotics. Furthermore, HGT equips *S. aureus* with potent virulence genes. This is evidenced by the fact that most virulence genes are encoded on MGEs. HGT is thus an important means for *S. aureus* to conquer the different niches in the human body and to achieve resistance to antibiotic therapy. In addition, HGT may provide *S. aureus* with advantageous metabolic features, as exemplified by the arginine catabolic mobile

element (ACME). This MGE is wide-spread among isolates of the *S. aureus* USA300 lineage, enhancing their potential for growth and survival[13].

Staphylococcal antibiotic resistance

Up until the discovery of the antibiotic penicillin by Sir Alexander Fleming in 1929, mortalities due to microbial infections were very high. Importantly, the introduction of penicillin into the clinic in 1940 reduced the mortality due to *S. aureus* bacteremia from 82% to less than 30%[14]. Sadly, *S. aureus* developed resistance against penicillin already two years after its clinical introduction. This resulted from acquisition of a plasmid carrying the *blaZ* gene, encoding a penicillinase that hydrolyzes the β-lactam ring of penicillin, thereby inactivating the drug. The expression of *blaZ* is under the control of two genes, *blaR1* that encodes a signal-transducing membrane protein, also known as an anti-repressor, and *blaI* that encodes a repressor protein. Upon exposure to β-lactams BlaR1 senses the β-lactams and activates a proteolytic domain, leading both to self-cleavage and cleavage of the BlaI repressor, allowing the expression of *blaZ*[15]*.* As a result, penicillin-resistant *S. aureus* started to rise in hospitals and was subsequently disseminated into the community, leading to a current prevalence of penicillin resistance in ~90% of the *S. aureus* isolates from humans[16,17].

The rise in penicillin-resistant *S. aureus* impelled the development of methicillin, a semi-synthetic derivative of penicillin, which was introduced in 1961. However, methicillin-resistant *S. aureus* (MRSA) strains emerged shortly following its introduction[18]. Acquisition of the *mecA* gene, encoding the penicillin-binding protein (PBP) 2a also called PBP2', is responsible for resistance to β-lactam antibiotics, including methicillin[19]. The *mecA* gene is located on a MGE called the Staphylococcal Cassette Chromosome *mec* (SCC*mec*). It is speculated that the first MRSA strain(s) acquired the *mecA* gene through HGT from coagulase-negative staphylococci. PBPs are membrane-bound transpeptidases needed for the synthesis of peptidoglycan, the main constituent of the cell wall of Gram-positive bacteria. While most PBPs are inhibited by β-lactam antibiotics, the active site of PBP2a has a lower affinity for these antibiotics allowing the synthesis of cell wall in their presence[20]. Unfortunately, the resistance of *S. aureus* is not limited to β-lactam antibiotics. Instead, this opportunist has developed resistances against essentially all antibiotics that were developed over the past decades including important last resort antibiotics, such as linezolid and daptomycin.

Staphylococcal virulence factors

The broad range of diseases caused by *S. aureus* mainly relates to the pathogen's broad arsenal of virulence factors. In order to effectively cause a disease, *S. aureus* has first to colonize the host, thereby evading the host immune responses[21,22]. Adherence of *S. aureus* to host tissues and cells is the first step in the infectious process that is facilitated by cell surface proteins, most of which were collectively termed 'microbial surface components recognizing adhesive matrix molecules', in short MSCRAMMs[23,24]. The MSCRAMMs are molecules that are exported from the cytoplasm with an N-terminal signal peptide. In addition, they have a C-terminal LPXTG motif and a hydrophobic membrane-spanning region followed by a positively charged residue. The LPXTG motif is a recognition sequence for sortase A (SrtA), a membrane-anchored transpeptidase. SrtA cleaves the LPXTG motif between the Thr and Gly residues and attaches, at the same time, the respective surface protein covalently to the cell wall[25,26]. Subsequently, the cell wall-bound MSCRAMMs facilitate the attachment of bacteria to plasma proteins or host extracellular matrices. Members of the MSCRAMM family include the fibronectin-binding proteins A and B (FnbpAB), the clumping factors A and B (ClfAB), the collagen binding protein (Cna), the SdrC and SdrD proteins that also bind fibrinogen, and the virulence-associated cell wall-anchored protein SasG[27].

In response to the bacterial presence, the host mounts innate and adaptive immune responses against the bacteria. To evade or fight the host immune responses, *S. aureus* has developed several mechanisms involving different cell surface-associated factors, such as the staphylococcal protein A (SpA), capsular polysaccharides, and the pigment staphyloxanthin that gives *S. aureus* its golden appearance, as well as a variety of secreted factors. SpA is a member of the MSCRAMM family that modulates the interaction of *S. aureus* with the von Willebrand factor (vWF), which allows *S. aureus* to adhere to platelets under shear stress conditions[28]. Furthermore, SpA has a high affinity for the Fc region of immunoglobulin-G (IgG)[29], and it can also bind the variable region of human IgG molecules from the VH3 family[28,30]. Consequently, SpA interferes with phagocytosis and binding of the complement system. Capsular polysaccharides help to impede phagocytosis by neutrophils thereby enhancing bacterial persistence[27,31]. Staphyloxanthin provides resistance against the potentially lethal effects of reactive oxygen species[32].

The secreted factors that allow *S. aureus* to survive insults from the host immune system can be divided into three categories, namely superantigens, cytolytic (pore-forming) toxins, and immune evasion factors (Fig. 1).

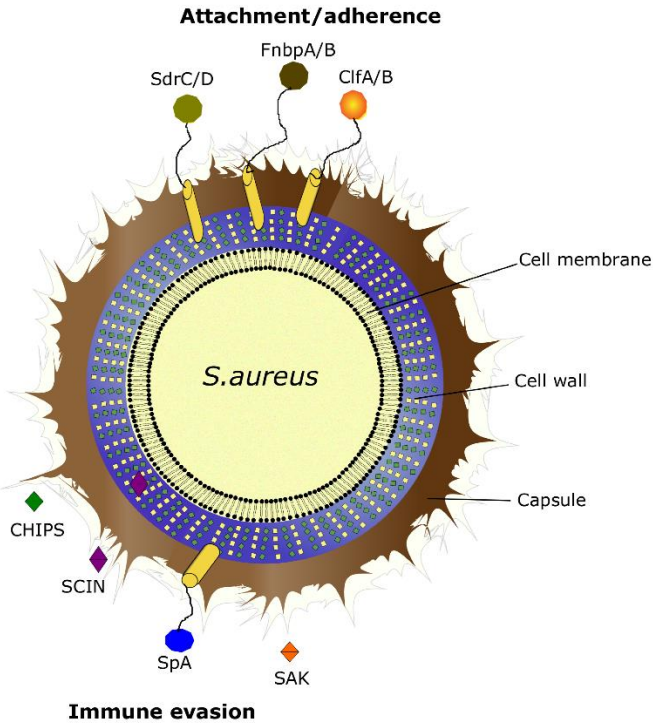

Figure 1. *S. aureus* proteins involved in attachment to the host and evasion of the immune defences. The *S. aureus* cytoplasm is enclosed by a cell membrane, cell wall, and capsule (from inside to outside). MSCRAMM proteins involved in the attachment to host cells and tissues, include FnbpA/B, ClfA/B, and SdrC/D. Other surface-attached proteins, such as SpA, or secreted proteins, such as the chemotaxis inhibitory protein of *S. aureus* (CHIPS), staphylococcal complement inhibitor (SCIN), and staphylokinase (SAK) are involved in evasion of the host immune defences. Yellow cylinders represent a cell wall-binding domain.

Superantigens are secreted proteins of *S. aureus.* They include four subclasses, namely i) enterotoxins for which more than 20 antigenic types are known (i.e. SEA to SEV)[33], ii) five exotoxin-like proteins (Set1 to Set5), iii) the exfoliative toxins (ETAs), and iv) the toxic shock syndrome toxin (TSST-1)[34,35]. These superantigens have been implicated in different diseases ranging from mild food poisoning (enterotoxins) to staphylococcal scalded skin syndrome (ETAs), a disease characterized by the loss of superficial skin layers, and toxic shock syndrome (TSST-1)[34–36]. They act by crosslinking the β-chain of T cell receptors and α and/or β chains of major histocompatibility class II molecules on the surface of antigen-presenting cells in an antigen-independent manner. Consequently, superantigens induce overproduction of cytokines and chemokines, and hyperactivate the immune system[37].

Cytolytic toxins contribute to immune evasion by damaging the plasma membrane of host cells. They form β-barrel pores or a short-lived pores in the cytoplasmic membranes of target cells, which causes either leakage of the cell's content or lysis of cells. This group of virulence factors includes on the one hand receptor-mediated pore-forming toxins, such as bi-component leukocidins (e.g. α-hemolysin), the Panton Valentine leukocidin (PVL), LukED, and LukGH (also known as LukAB), and γ-hemolysin. On the other hand, this group includes non-receptor mediated toxins, such as the α-type phenol soluble modulins (PSMα1-4)[38,39] (Fig. 2). The receptor-mediated binding of α-hemolysins to host cell membrane creates first a pre-pore, which then matures into a β-barrel transmembrane pore that allows a loss of molecules smaller than 2 kD[40], leading to death of the host cell. In contrast, the formation of a mature pore by bi-component leukocidins requires two polypeptides referred to as S (slow) and F (fast). As such, HlgA or HlgC, and HlgB belong to the γ-hemolysin S and F components, respectively[38,41]. Similarly, PVL consists of S and F components encoded by the two co-transcribed phage-borne genes *lukS*-PV and *lukF*-PV[42]. The mode of action of the bi-component toxins is similar to that of α-hemolysins, but the lytic ability of these toxins is species- and cell-specific[43].

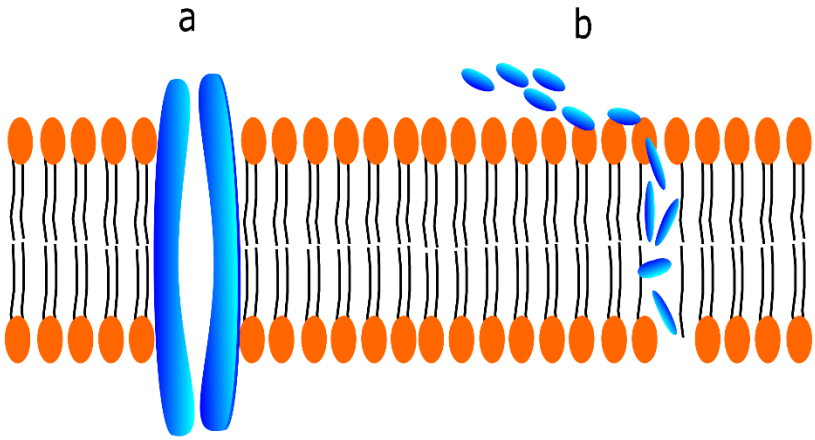

Figure 2. Schematic representation of host membrane damage by staphylococcal toxins. *S. aureus* toxins, such as α-hemolysins, PVL, LukAB (LukGH), and LukDE form pores in the host cell membrane through which essential ions and metabolites are lost (a), whereas PSMα type toxins attach to the host cell membrane and perturb its integrity leading to cytolysis (b). Image adapted from Otto M[39].

Vandenesch *et al.* reported PVL as a common genetic marker for community-associated (CA) MRSA lineages[44] that were associated with skin and soft tissue infections. However, subsequent studies showed the emergence of CA-MRSA isolates that lack PVL[45–47]. Of note, the role of PVL in virulence has been debated on the basis of *in vitro* and *in vivo* experiments[48–51]. *In vitro*, murine neutrophils were shown to be insensitive to PVL, while

human and rabbit neutrophils showed a high sensitivity to PVL. On the other hand, several association studies to assess the role of PVL in human pathogenesis yielded inconsistent results. For instance, a meta-analysis of 76 studies showed no association between PVL and increased mortality, prolonged hospitalization, or increased disease severity[52]. On the other hand, a study conducted in a Chinese hospital reported that patients infected with hospital-associated *S. aureus* carrying PVL displayed more-severe disease symptoms and died earlier[53]. Consistent with the *in vitro* data, the effects of PVL in animal infection models varied. In murine sepsis and abscess models, there was no significant difference observed in the survival of mice infected with *S. aureus* USA300 or USA400 wild-type strains and their isogenic mutants lacking PVL[54]. In contrast, rabbits displayed a higher sensitivity to PVL. However, rabbits also suffered from hypersensitivity to particular toxins, such as the α-toxin of *S. aureus*. In fact, these observations highlight the difficulty in finding appropriate animal models to investigate *S. aureus* infections[55]. Recently, it was reported that PVL is host cell type- and host species-specific, binding preferably to the human version of its receptor, the C5aR protein[56]. Therefore, it is important that advances have been made over the past three years in the development of a humanized mouse model to study *S. aureus* infection *in vivo*[57–59]. To this end, the authors used non-obese diabetic gamma (NSG) mice with severe combined immune deficiency (scid). These immune-deficient mice lack B and T cells as well as natural killer cells, and they have defective myeloid cells. Instead, they were provided with a human hematopoietic system through fetal hematopoietic stem cell (CD34+) and thymic tissue grafts. Analyses with these humanized mice showed the role of PVL both in an *S. aureus* skin infection model[58] and a pneumonia model[59] through the use of PVL-positive MRSA strains and their isogenic PVL mutants. In these studies, severe pathological conditions were observed in the NSG mice infected with PVL-positive strains of MRSA compared to the non-humanized mice. It thus seems that the previous controversy on the role of PVL in *S. aureus* pathogenesis is attributed to its species-specificity.

PSMs were first identified in *Staphylococcus epidermidis* with a designation as pro-inflammatory complex[60]. Subsequently, they were identified in *S. aureus* and shown to have a cytolytic activity towards neutophils[61]. There are four types of PSMs known in *S. aureus*, namely the PSM*α,* PSMβ*,* PSMγ and PSM-*mec*. PSMα 1-4 are encoded by the *psmα* locus, PSMβ1 and PSMβ2 are encoded by the *psmβ* locus, PSMγ is encoded by RNAIII specified by the *agr* locus[61], and PSM-*mec* is encoded by SCC*mec* types II, III, and VIII MGEs[62,63]. The α-type peptides share a relatively small size with about 20-25 amino acids while the β-type peptides are about 44 amino acids long. Their ability to lyse

neutrophils has been extensively investigated since their discovery in 2007[61]. Of note, this lytic activity is restricted to the PSMα proteins with a most pronounced effect of PSMα3[61,63]. In addition to their cytolytic activity, PSMα contributes to the escape from phagosomes in both non-professional and professional phagocytic cells and, consequently, to the evasion of killing by immune cells of the host[64]. In a murine *S. aureus* skin infection model, there were no differences observed in the virulence of wild-type strains with *psm-mec* and their isogenic deletion mutants[65]. This is consistent with a study showing a lower cytolytic activity of PSM-*mec* compared to the other PSMs. However, PSM-*mec* enhances the cytolytic potential of *S. aureus* strains that produce relatively low amounts of the other PSMs[63]. Furthermore, the PSMs, in particular PSMα3 and PSMγ, were implicated in spreading of *S. aureus* over wet surfaces and biofilm dynamics[66].

The third group of secreted immune evasion factors of *S. aureus* consists of MGE-encoded proteins with various activities, such as staphylokinase (SAK), the chemotaxis inhibitory protein of *S. aureus* (CHIPS), the staphylococcal complement inhibitor (SCIN), the extracellular fibrinogen binding protein (Efb), and the extracellular adherence protein (Eap) (Fig. 1). SAK binds to α-defensins, which are bactericidal peptides produced by human neutrophils, resulting in the inactivation of their antimicrobial activity[67,68]. In addition, SAK binds to human plasminogen resulting in the activation (conversion) of plasminogen into plasmin at the surface of the bacteria. Hence, SAK creates a bacteria-bound serine protease activity that results in cleavage of IgGs and the C3b complement, thereby exerting an anti-opsonic activity and preventing phagocytosis of the bacteria[69]. CHIPS, SCIN, Efb, and Eap inhibit activation of neutrophils and the complement system, and neutrophil chemotaxis[70–72]. Of note, while SCIN is generally regarded as a secreted protein, a recent study has shown that it is recruited to the *S. aureus* cell surfaces by human C3 convertases whose activity is inhibited by SCIN[73].

Altogether, the different surface-associated and secreted virulence factors of *S. aureus* facilitate the attachment to, invasion of, and lysis of human host cells, and they allow the inactivation and evasion of the host immune defenses, thereby establishing the symptoms of disease. In order to accomplish these activities, *S. aureus* selectively modulates the activities of particular groups of genes that are controlled by different global regulators.

Regulation of staphylococcal virulence factors

The regulation of bacterial gene expression is growth phase- and/or growth condition-specific and, to a large extent, it involves transcriptional regulation. In *S. aureus*, two-component gene regulatory systems, such as AgrAC, ArlSR, HssRS, LytRS, SaeRS, and

SrrAB are the main regulators of virulence genes[74]. Additionally, virulence genes are regulated by the alternative sigma factor SigB and DNA-binding proteins, such as SarA and its homologues Rot, SarR, SarS, SarT, and SarU[74].

Two-component regulatory systems are responsive to different environmental signals, such as bacterial cell density, pH, CO_2, and nutrient availability. They consist of a sensor histidine kinase and a response regulator. In general, the induction of autophosphorylation of the histidine kinase by environmental stimuli initiates a cascade of phosphorylation reactions, leading to the phosphorylation of the cognate response regulator. Depending on its phosphorylation state, the response regulator binds to a particular DNA region, thereby either enhancing or repressing expression of the respective gene, or genes in case of operon-like structures[75].

As detailed in chapters 3 and 4 of this thesis, a large number of genes and proteins are under the control of the Agr and SigB regulatory systems[76]. Therefore, the regulatory mechanisms employed by these two systems are discussed in more detail here.

The accessory gene regulator (Agr) locus encodes two divergent transcripts, RNAII and RNAIII, which are controlled by the P2 and P3 promoters, respectively. RNAII encodes AgrB, AgrD, AgrC and AgrA that are components of the staphylococcal quorum-sensing system[77]. AgrC and AgrA function as sensor and response regulators, respectively. The *agrD* transcript encodes the pro-peptide of an autoinducing peptide (AIP) that requires maturation and secretion into the extracellular environment, a process carried out by the membrane-anchored protein AgrB. In order for AIP to be functional, AgrB first introduces a thiolactone modification (i.e. a thioester bond between the sulfhydryl group of a cysteine residue and the C-terminal carboxyl group) in the AgrD pro-peptide, followed by C-terminal cleavage, and export of the AgrD-derived AIP[78,79]. Of note, there are four allelic variants of *agr* known, named types I-IV, which encode distinct AIPs that serve as specific activating ligands for particular AgrC types, while inhibiting other variants of AgrC[80]. The cognate extracellular AIP activates the two-component AgrC-AgrA system in a cell density-dependent manner. Upon binding of AIP to AgrC, this transmembrane AIP receptor becomes phosphorylated[81]. Subsequently, the phosphate is transferred from AgrC to AgrA, promoting the binding of this response regulator to the intergenic region between the P2 and P3 promoters, leading to the synthesis of RNAII and RNAIII[82]. As indicated above, the Agr effector RNAIII encodes the δ-hemolysin (also known as PSMγ). RNAIII also controls the expression of various genes for virulence factors by governing the switch between expression of cell-surface associated proteins genes and secreted proteins[83]. In addition to binding to the intergenic region of P2 and

P3, phosphorylated AgrA binds and activates the promoters that regulate the expression of the genes that encode PSMα and PSMβ[84] (Fig. 3). Altogether, the regulation of the Agr operon depends on the density of the *S. aureus* population, which ultimately determines whether particular virulence genes are either expressed or repressed.

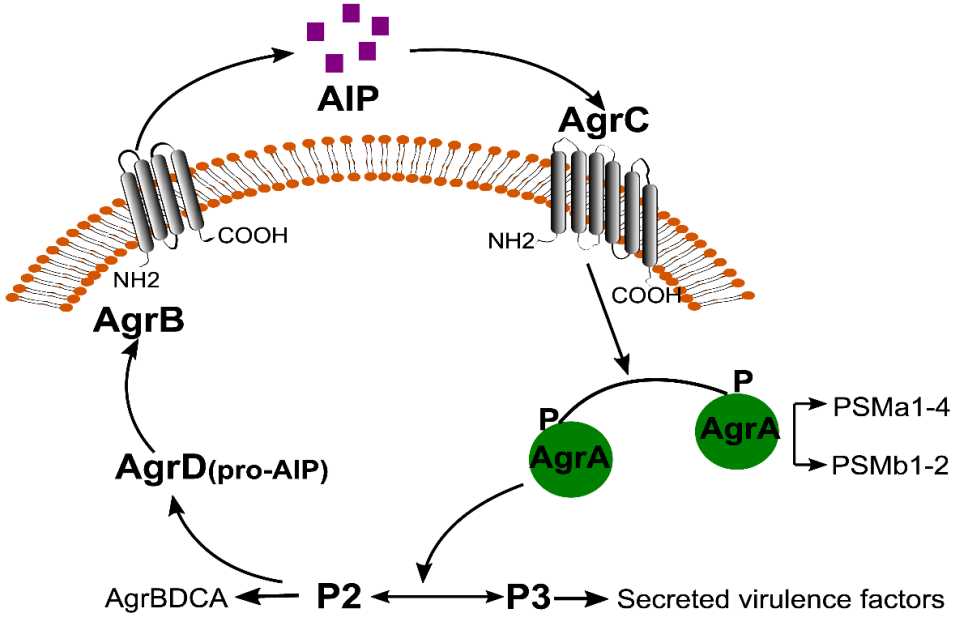

Figure 3. The Agr quorum-sensing system of *S. aureus*. AgrD is the pro-peptide of the autoinducing peptide (AIP). The membrane-bound AgrB protein is responsible for modification, maturation and secretion of AIP. The secreted AIP binds to the cognate AgrC, modulating the autophosphorylation of AgrC which, subsequently, leads to transfer of the phosphate to AgrA. The phosphorylated form of AgrA then triggers activation of the P2 and P3 promoters that control expression of the *agr*-operon and RNAIII-encoding PSMγ. In turn, RNAIII induces the expression of different virulence factors, including PSMα and PSMβ. Figure adapted from Painter KL *et al.* [85].

The alternative sigma factor B (SigB) of *S. aureus* may act independently or in cooperation with other regulators. Compared to the Gram-positive bacterium *Bacillus subtilis*, relatively little is known about the signal perception by SigB in *S. aureus*. The *sigB* operon of *B. subtilis* contains eight genes, of which only four, namely *rsbU*, *rsbV*, *rsbW,* and *sigB* (*rsb* stands for **r**egulator of **s**igma **B**) are conserved in *S. aureus*[86]*. rsbU, rsbV, rsbW* tightly regulate SigB. Of note, the regulatory mechanism of SigB in most bacteria follows the same principle, involving multiple protein-protein interactions triggered by different stress conditions. Normally SigB exists in an inactive state by forming a complex with its antagonist, the anti-sigma factor RsbW. Under these conditions RsbV (anti-anti sigma factor) is phosphorylated and thereby inactivated by RsbW. However, following particular environmental stimuli, RsbV-P is dephosphorylated

by RsbU and then active. RsbW is bound by non-phosphorylated RsbV, which frees SigB and allows it to interact with an RNA polymerase, an event that consequently leads to transcription of SigB-dependent genes[87,88] (Fig. 4). However, it is not clear which signals are perceived by RsbU to activate its phosphatase activity. Furthermore, when heterologously expressed in *B. subtilis, S. aureus´* RsbU is permanently active in contrast to its *B. subtilis* homolog[88].

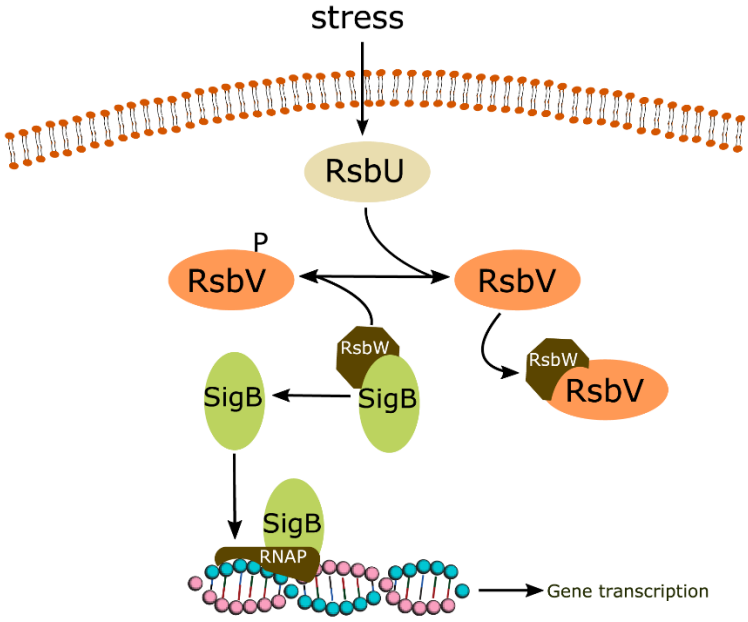

Figure 4. **Mechanism of SigB-dependent gene regulation in *S. aureus***. Upon sensing of environmental stress stimuli, the RsbU protein dephosphorylates RsbV. Dephosphorylated RsbV then binds to RsbW, resulting in the release of SigB that has been sequestered by RsbW. As a consequence, free SigB will bind to RNA polymerase (RNAP) allowing the transcription of genes belonging to the SigB regulon. Phosphorylated RsbV is inactive, and it cannot bind RsbW. In fact, RsbW promotes the phosphorylation of RsbV in order to keep RsbV in an inactive state. Figure is adapted from Junecko JM *et al.*[74].

In addition to Agr and SigB, *S. aureus* contains many more regulators of virulence gene expression. Notably, these different regulators do not only function directly in activating or repressing virulence genes, but they also exert indirect effects by influencing each other's activity. Such regulatory networks of *S. aureus* have been reviewed by Priest *et al* [89]. Furthermore, a web-based resource called regprecise (http://regprecise.lbl.gov/RegPrecise/index.jsp) provides overviews of gene regulatory cascades in different taxa including staphylococcaceae[90] (Fig. 5). Of note, natural 'mutations' in diverse regulators can occur, and the respective isolates may show differential behavior with respect to virulence or resistance to antibiotics. This makes it

difficult to predict the virulence of different types of *S. aureus* isolates based on only the genome sequence.

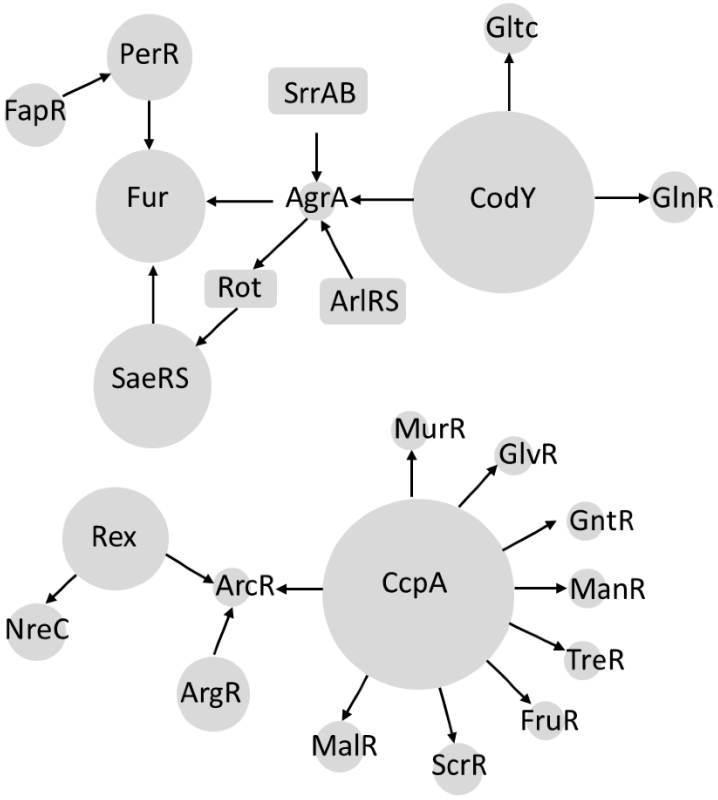

Figure 5. Inter-regulatory networks of *S. aureus* regulons. Particular regulators may either promote activation or repression of other regulators and their regulons. In the present Figure, each circle/rectangular box represents a particular regulon known to control one or more *S. aureus* regulators and their regulons. Arrow heads indicate regulons with a dominant regulatory effect over other regulons. The sizes of the circles are proportional to the numbers of genes that are under the control of a particular regulator or regulon, with larger circle sizes indicating larger numbers of genes. Rectangular boxes represent regulons with unknown number of target genes. The figure was adapted from the regprecise website[90] and Priest NK *et al.*[89].

Molecular typing of *S. aureus*

Molecular typing of microbial pathogens, such as *S. aureus*, provides important information about i) genetic microvariations that support the investigation, control, and prevention of outbreaks in hospitals and the community, and ii) genetic macrovariations that guide phylogenetic and population-based analyses[91]. Classical molecular typing techniques are gel-based, as exemplified by 'pulsed-field gel electrophoresis' (PFGE), 'multi-locus variable number tandem repeat analysis' (MLVA), and 'multiple-locus variable number tandem repeat fingerprinting' (MLVF). The more advanced typing

techniques are sequence-based, and address either a single locus (e.g. *spa*-type), multiple loci (e.g. 'multilocus sequence typing' [MLST]), or whole genome sequences (WGS).

PFGE is a typing method that analyzes a fragment of DNA generated by digesting bacterial chromosomes with the restriction enzymes *Sma*I or, in case of livestock-associated MRSA, *Ehe*I. The digestion products are then separated on an agarose gel with alternating pulses of current that change the orientation of the electric field across the gel[92]. Based on the banding patterns observed upon PFGE, isolates are assigned to a specific PFGE profile (e.g. *S. aureus* USA200, USA300, USA500, etc.). PFGE is still considered as the 'gold standard' typing method due to its high discriminatory power. However, it is labor-intensive and a direct comparison of results obtained in different laboratories is difficult[93].

MLST involves the amplification and subsequent sequencing of 450-500 bp stretches of seven *S. aureus* housekeeping genes, namely *arcC*, *aroE*, *glpF*, *gmk*, *pta*, *tpi,* and *yqiL*[94]*.* The resulting sequences are compared with sequences deposited in the online MLST web server (http://www.mlst.net/), on which basis a number is given to each locus, resulting in a seven-digit allelic profile. As such, MLST allocates *S. aureus* isolates to different sequence types (STs). In addition, MLST data can be used in the 'based upon related sequence types' (BURST) clustering algorithm to group strains into clonal complexes (CCs)[95,96]. As MLST is based on sequencing, the results obtained with this method in different laboratories can be readily compared. However, it was thus far relatively expensive and labor intensive to sequence seven genes[95]. Also, it only addresses variations in the core genome, while the largest variations between isolates occur in the accessory genome.

spa-type is based on the gene encoding the above-mentioned cell wall-associated SpA protein of *S. aureus*[97]*.* In its C-terminus SpA has a variable region (X), and sequencing of the respective variable gene region unveils extensive polymorphisms, which allow the grouping of isolates into different *spa*-types[97,98]. To this end, the specific *spa* gene sequence of an isolate is compared with sequences deposited to the online *spa*-server (http://www.spaserver.ridom.de/), resulting in the assignment of a *spa*-type. The fact that this typing technique only addresses a single locus makes it less expensive and less labor intensive. However, it has a relatively moderate discriminatory power as different *S. aureus* lineages as defined by MLST can have the same or similar *spa* loci[99].

Typing methods based on 'variable number tandem repeats' (VNTRs), such as MLVA and MLVF, essentially assess regions of coding and non-coding nucleotide repeats.

Specifically, MLVA determines the numbers of VNTRs at eight different loci[100]. It involves the amplification of the selected VNTR loci by multiplex PCR followed by agarose gel electrophoresis to separate the amplified loci or, more recently, by automated fragment sizing of these loci on a DNA sequencer. The results are used to calculate the number of repeats in each VNTR locus with specific software. The numbers of repeats of the eight VNTR loci are then combined to form an MLVA profile (e.g. 14-0-2-4-1-7-1-6)[100,101]. MLVA has similar discriminatory power as PFGE, but it is less tedious and much faster than PFGE.

MLVF is a simple typing method based on the amplification and electrophoretic separation of repeated sequences in seven selected genes, namely *sspA*, *spa*, *clfA*, *clfB, sdrC*, *sdrD*, and *sdrE*[102]. A limitation of this technique is the difficulty to assign amplified fragments to the corresponding target genes. Since it is not possible to correctly analyze the number of repeats in each amplicon, it is a genuine 'fingerprinting' technique[99,103]. Yet, MLVF is cheaper, faster, and much easier to implement than other typing methods, and it has a higher discriminatory power. A major limitation is that inter-laboratory comparison of MLVF data is impossible.

Lastly, *S. aureus* isolates are often characterized by PCR-based identification of specific virulence-associated genes, such as the PVL genes, SCC*mec*[104], *agr,* and prophages[105]. Importantly, the recent advancements in the availability and affordability of WGS have created a most favorable platform for the typing of microorganisms in general, and *S. aureus* in particular[106]. Altogether, the replacement of gel-based by sequence-based typing methods has many merits, not in the last place high inter-laboratory comparability and a major improvement in the discriminatory power[106,107].

Molecular epidemiology of MRSA

The first report of MRSA came from a British hospital in 1961. Since then, MRSA has spread around the world, becoming a major public health problem that causes both hospital-associated (HA) and community-associated (CA) infections. The traditional classification of CA- and HA-MRSA by the Active Bacterial Core Surveillance Program of the Centers for Disease Control and Prevention in the USA defines an *S. aureus* isolate as CA-MRSA when the respective patient did not undergo hemodialysis, surgery, hospitalization, or had no history of residence in long-term care facilities within the year preceding its isolation and previous MRSA infection, or was not hospitalized >48hrs before the positive MRSA test. In contrast, isolates from patients with such a clinical history are defined as HA-MRSA[108]. CA-MRSA lineages predominantly cause skin and soft tissue infections, but they may also cause severe invasive disease among immune-competent people. On the other hand, HA-MRSA lineages mainly cause bloodstream

infections in immune-suppressed individuals[109]. Until today, antibiotic resistance profiles, SCC*mec* typing, PVL, and the superantigen status are being used to distinguish CA- and HA-MRSA as a package for control and prevention of outbreaks. However, the distinction between CA- and HA-MRSA isolates remains difficult as the criteria to distinguish such isolates are relatively soft. Consequently, there is a clear need to better understand the distinctive molecular features that dictate the differences in epidemiology of CA- and HA-MRSA.

Scope of the thesis

The research described in this dissertation was aimed at identifying possible links between staphylococcal epidemiology and pathophysiology. A notorious lineage of CA-MRSA is represented by *S. aureus* with the PFGE type USA300, the MLST type ST8 and the *spa*-type t008, which was first identified from community-acquired infections in the USA[110,111]. In Europe, the USA300 lineage was first recognized among Danish patients in 2000, followed by patients in many other European countries[112,113]. Interestingly, nowadays also HA-associated infections with MRSA of the USA300 lineage are encountered. This provided an opportunity to investigate distinguishing molecular features of closely related CA- and HA-MRSA isolates through a comparative genome, transcriptome, and proteome analysis. In particular, the present studies were focused on Danish *S. aureus* USA300 isolates[112,113]. Specifically, the investigated CA-USA300 isolates from Denmark belong to the sequence type ST8 and the *spa*-type t008, are PVL-positive and carry the ACME element, while the investigated HA-USA300 isolates belong to ST8 and *spa*-type t024, are PVL-negative, and carry in most cases the ACME element. The essential background information for this research with respect to staphylococcal MGEs, virulence, gene regulation and epidemiology is summarized in **Chapter 1** of this thesis.

Chapter 2 presents global distinguishing features of CA- and HA-MRSA isolates of the USA300 lineage through comparative genome and exoproteome analyses. In brief, the comparative genome analyses revealed a distinct clustering of the CA- and HA-MRSA that was mirrored in the respective profiles of secreted proteins and survival inside human epithelial cell lines.

The vast majority of proteins that were identified in the exoproteome analyses described in chapter 2 were predicted as cytoplasmic proteins. Since these proteins were important for the distinction of CA- and HA-MRSA isolates, it was important to further investigate whether this distinction would also be reflected in cytoplasmic proteins extracted from the respective bacterial cells. Therefore, cytoplasmic proteins from cells of both groups

of MRSA isolates were investigated by mass spectrometry. Indeed, as described in **Chapter 3,** the proteomics analysis of the cytosolic cell fraction allowed a clear distinction of CA- and HA-MRSA isolates. In particular, the cytoplasmic proteins of the CA- and HA-MRSA isolates uncovered noteworthy differences in central carbon metabolism that apparently match with the clinical presentation of the respective groups of isolates.

Since bacterial gene regulation occurs to a large extent at the transcriptional level, the molecular distinction of the investigated CA- and HA-USA300 isolates was extended with an RNA sequencing analysis. The results of this analysis are described in **Chapter 4**. Importantly, the outcomes indicated potential differences in the ability of the CA- and HA-USA300 isolates to survive phagocytosis, and this was subsequently verified experimentally in human neutrophils and hemocytes (i.e. phagocytic cells) from the infection model *Galleria mellonella*. Altogether, the results imply that prolonged intra-phagocyte survival reflects a strategy of *S. aureus* USA300 to adapt to the hospital setting where the antibiotic pressure is much higher than in the community.

Chapter 5 summarizes the main findings and conlusions presented in this thesis, and it describes the perspectives for future research on the distinctive features of CA- and HA-MRSA. In particular, chapter 5 reflects on how the present findings can be applied towards the development of diagnostic markers to distinguish CA- and HA-MRSA for epidemiological investigations and, most importantly, for the control and prevention of outbreaks.

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